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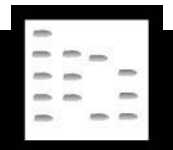
FORENSIC TOXICOLOGY: THIN-LAYER (PLANAR) CHROMATOGRAPHY



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The selection of appropriate analytical methodology for forensic toxicological investigations depends on the scope of the laboratory. Postmortem forensic toxicology investigates the cause of death, and consequently a very broad-range screening is needed to detect all potential poisons. In traffic toxicology, only such substances are relevant which may impair the driver's ability to control the vehicle. Doping control focuses on those substances that have been banned by the International Olympic Committee. Prisoners and rehabilitation clinic patients are tested for psychotropic drugs, whereas the US Mandatory Guidelines for Federal Workplace Drug Testing Programs are limited to the major drugs of abuse, cannabis, cocaine, amphetamine, opiates and phencyclidine.

Thin-layer chromatography (TLC) has found extensive use in forensic toxicology since the early 1960s when the famous book of Stahl made the technique well known. The first edition of the classic laboratory manual by AS Curry, *Poison Detection in Human Organs* from 1963 (Charles C. Thomas, Springfield, IL), still relies on paper chromatography but the second edition in 1969 utilizes TLC as a major technique for drugs. Gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS) in the 1970s, and especially high performance liquid chromatography (HPLC) in the 1980s gradually began to replace TLC but today the planar technique is having a renaissance due to the progress in instrumentation and software. From 1990 to 1996, 26% of published TLC applications were in the field of medical, clinical and biological analysis, which also includes forensic toxicology.

The commonly recognized advantages of classical manual TLC are high throughput, low cost, easy sample preparation and versatile visual detection possibilities. Instrumental TLC extends the scope to reproducible quantitative analysis and allows the utilization of *in situ* UV spectral information for identification. The main disadvantage of TLC is low chromatographic resolution, which can be partly overcome by instrumental techniques. Another disadvantage is that quantitative calibration curves are not reproducible enough to be stored, making it necessary to co-analyse several standards along with samples on each TLC plate. Most of the substances frequently encountered in forensic toxicology can be readily analysed by TLC. These include therapeutic drugs, drugs of abuse, pesticides and naturally occurring alkaloids, which are all relatively small molecular weight organic compounds with functional groups amenable to visualization by colour reactions.

It is practical to divide the discussion of TLC in forensic toxicology into two categories, the broad-scale screening analysis and target analysis. The former approach is related to the concepts of systematic toxicological analysis or general unknown, i.e. the search for a rational qualitative analysis strategy for hundreds of potential poisons. TLC drug screening is often performed in urine or liver, where the drug concentrations are higher than in the blood. In target analysis, the aim is specifically to detect and often also to quantify a substance or a limited number of substances.

Broad-scale Screening Analysis

Chromatographic Systems

Evaluation of systems The rational selection of TLC systems for screening analysis differs from the optimization of the separation of a few-component

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mixture. In screening systems, the most important features are the distribution of R_F values across the plate, the reproducibility of the measurement of those values, and the correlation of chromatographic properties between systems. The computational methods capable of taking into account these features include discriminating power, mean list length (MLL), information content, quotient of distribution equality and principal component analysis. The (MLL) method has found widespread use, and it can also be used in computerized substance identification. The MLL approach is not related to the separation number (SN), i.e. the number of spots that can be separated by a system with a certain resolution. **Table 1**

Table 1 TLC systems for broad-scale toxicological screening analysis

	Mobile phase	*Stationary phase*	*Correction standards*	hR_F^c	*Application*
1	Chloroform–acetone 80 + 20	Silica gel	Paracetamol Clonazepam Secobarbital Methylphenobarbital	15 35 55 70	Acidic and neutral drugs
2	Ethyl acetate	Silica gel	Sulfathiazole Phenacetin Salicylamide Secobarbital	20 38 55 68	Acidic and neutral drugs
3	Ethyl acetate–methanol–conc. ammonia 85 + 10 + 5	Silica gel	Hydrochlorothiazide Sulfafurazole Phenacetim Prazepam	11 33 52 72	Acidic and neutral drugs
4	Methanol–water 65 + 35	Silica gel RP 18	Diazepam Secobarbital Phenobarbital Paracetamol	16 35 54 74	Acidic and neutral drugs
5	Methanol–water–conc. hydrochloric acid 50 + 50 + 1	Silica gel RP 18	Hydroxyzine Lignocaine Codeine Morphine	20 46 66 81	Basic, amphoteric and quaternary drugs
6	Toluene–acetone–ethanol–conc. ammonia 45 + 45 + 7 + 3	Silica gel	Codeine Promazine Clomipramine Cocaine	16 36 49 66	Basic and neutral drugs
7	Ethyl acetate–methanol–ammonia 85 + 10 + 5	Silica gel	Morphine Codeine Hydroxyzine Trimipramine	20 35 53 80	Basic and neutral drugs
8	Methanol	Silica gel	Codeine Trimipramine Hydroxyzine Diazepam	20 36 56 82	Basic and neutral drugs
9	Methanol–ammonia 100 + 1.5	Silica gel[a]	Atropine Codeine Chlorprothixene Diazepam	18 33 56 75	Basic and neutral drugs
10	Cyclohexane–toluene–diethylamine 75 + 15 + 10	Silica gel[a]	Codeine Desipramine Prazepam Trimipramine	6 20 36 62	Basic and neutral drugs

[a]Impregnated with 0.1 mol L^{-1} KOH and dried.

shows TLC systems for broad-scale toxicological screening analysis, chosen partly on grounds of the MLL method, while the corresponding R_F libraries can be found from the books of de Zeeuw *et al*. and Fried and Sherma. For acidic and neutral drugs, recommended combinations of systems which posses low mutual correlation are 2 and 3, and 1 and 3, for basic drugs 5 and 6, and 8 and 10.

R_F correction In screening analysis, where R_F libraries of hundreds of compounds are utilized, the reproducibility of the values is an essential factor. TLC is an open technique, and the R_F values, and consequently the separation, are affected by environmental factors, such as humidity, layer activity and temperature. In contrast to column chromatography, the use of a single R_F standard for compensating the variation, corresponding to the relative retention time, may produce erroneous results. The method, which uses three to five correction standards that are structurally close to the analytes and linear interpolation between the standards, is now generally accepted to obtain corrected R_F values (hR_F^c). **Table 1** indicates the correction standards chosen for the screening systems listed.

Identification

Migration distance By carefully adjusting the chromatographic and environmental conditions it is possible to obtain reproducible results with precoated plates. Reversed-phase (RP) layers show more batch-to-batch variation than silica gel but RP separations, using aqueous mobile phases, are less dependent on humidity. The R_F and hR_F^c values can be determined manually or by using a scanning densitometer. The correction of R_F values makes it possible to obtain reproducible values in varying conditions and allows the use of the large hR_F^c libraries even in interlaboratory use.

Commercial software are available that utilizes the concept of hR_F^c for identification. Chrom TOX (Merck Tox Screening System, Merck, Darmstadt, Germany) is statistical search software that utilizes the MLL method for identification by R_F values and digitally coded colour reactions, giving a hit list of candidates with probability values. The software is also capable of adding information from other analytical techniques, such as retention indices from GC, molecular weights from MS and UV spectra from HPLC. A drawback is that the TLC data have to be fed manually. CATS software (Camag, Muttenz, Switzerland) combines instrumental densitometric evaluation of chromatographic plates with substance identification by hR_F^c values and *in situ* UV spectra (see below).

Visualization reagents The possibility of using visualization reagents for the detection and identification of fractions is a unique feature of TLC. Post-chromatography derivatization by spraying or dipping has been used more extensively than pre-chromatography derivatization. The limits of detection by colour reactions generally range from 0.1 to 1 μg per fraction and by fluorescence reactions,

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Table 2 Visualization reagents for broad-scale screening analysis

Reagent	*Application*
Bratton-Marshall reagent (diazotation and coupling)	Benzophenones (from benzodiazepines), sulfonamides
7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)	Amphetamines, amino acids
2,6-Dibromoquinone-4-chlorimide (Gibbs reagent)	Pesticides
2,6-Dichlorophenol-indophenol (Tillmann's reagent)	Organic acids
p-Dimethylaminobenzaldehyde (Van Urk's reagent)	Drugs, sulfonamides, pesticides
Dragendorff's reagent	Drugs and alkaloids
Fast Black K salt	Amphetamines, adrenergic β-blocking drugs, nor-metabolites
Fast Blue B salt, Fast Blue BB salt	Cannabinoids
Fluorescamine	Amphetamines, amino acids, sulfonamides
Forrest reagent	Phenothiazines, antidepressants
FPN reagent	Phenothiazines, dibenzazepines
Furfuraldehyde	Carbamates, phenothiazines
Iodoplatinate, acidic	Alkaloids, drugs, quaternary ammonium compunds
Mandelin's reagent	Drugs
Marquis reagent	Drugs
Mercuric chloride-diphenylcarbazone	Barbiturates
Mercurous nitrate	Barbiturates
Ninhydrin	Amphetamines, amino acids
4-(4-Nitrobenzyl)pyridine-tetraethylenepentamine	Pesticides
Salkowski reagent ($FeCl_3 + H_2SO_4$)	Phenothiazines, thioxanthenes
3,3′,5,5′-Tetramethylbenzidine, *o*-tolidine, after Cl_2	Pesticides, acidic and neutral drugs

especially using pre-chromatography derivatization, 20–100 ng per fraction. The visualization reactions can be divided into class and substance selective. In visualization sequences, several reagents can be oversprayed one after another to amplify the amount of information obtained from a single plate. An example of such sequence for basic drugs is ninhydrin, FPN ($FeCl_3 + HClO_4 + HNO_3$) reagent, Dragendorff's reagent and acidified iodoplatinate. **Table 2** lists reagents that are commonly used in forensic toxicology.

***In situ* spectra** Earlier it was common to scrape off a separated fraction from the TLC plate and submit it to a further spectrometric or spot test analysis. Today it is possible to measure the *in situ* UV spectrum of a fraction and compare this with stored spectrum libraries for identification. Representative spectra can generally be obtained from well-separated fractions with substance amounts over 0.5 μg on standard TLC plates and over 0.1 μg on high performance thin-layer chromatography (HPTLC) plates, although these limits depend on the shape of the fraction and on the absorption characteristics of the compound in question. The upper limit is not a problem as the reflectance saturates after certain level and the spectra remain practically the same. The CATS software from Camag allows the complete sequence of instrumental screening analysis, including R_F correction, spectrum measurement, automated search against hR_F^c/UV libraries and reporting (**Figure 1**).

Other spectrometric techniques than UV have been tested for the measurement of TLC fractions. *In situ* diffuse reflectance Fourier transform infrared (FTIR) measurements have proved to be feasible for the identification of drugs using a spectral region where silica gel has no strong absorption, and a commercial TLC-FTIR interface is available.

Proprietary Drug Screening Schemes

Particularly in North America, a TLC scheme called Toxi-Lab (Ansys, Irvine, CA, USA) has gained popularity in analytical toxicology. This product comprises the extraction, development, visualization and interpretation steps of analysis. The Toxi-Lab A detects basic and neutral substances and the Toxi-Lab B detects acidic and neutral substances. The plates consist of silica, impregnated with a vanadium salt for detection purposes, in a glass fibre matrix. The Toxi-Gram C8 are octylsilica bonded phase plates for the confirmation of basic and neutral drugs. All the plates have holes at the origin for the inoculation of factory-made standard substance discs and discs containing the evaporated sample residues. Detection is carried out by using a standardized four-stage

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Library: C:\CAMAG\KRIM1.SCL

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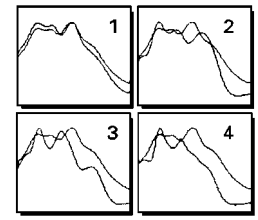
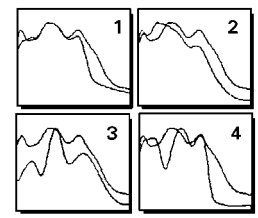


No.	Substance name	Diff	Correlation
1	Clozapine	2	0.984415
2	Thiothixene	− 9	0.948759
3	Norchlorprothixene	0	0.915627
4	Flupenthixol	− 1	0.902411
5	Olanzapine	− 1	0.891046
6	Norlevomepromazine	− 3	0.877048

Confirmation: ☐ necessary ☐ not necessary
Hit #_____ confirmed by A:_____ B:_____

Method: C:\CAMAG\DATA_SC3\RPSPEC.PAM
Raw data: C:\CAMAG\DATA_SC3\RY140798.DFS
Library: C:\CAMAG\RP1.SCL

Track 15, Analysis n: 2561
Peak #2, Measured hR_f^c: 46, Area: 13178.0



No.	Substance name	Diff	Correlation
1	Clozapine	3	0.940416
2	Clothiapine	− 9	0.937639
3	Molindone	− 9	0.893862
4	Brucine	− 4	0.868569
5	Apomorphine	2	0.857298
6	Metoclopramide	− 6	0.855726

Confirmation: ☐ necessary ☐ not necessary
Hit #_____ confirmed by A:_____ B:_____

Figure 1 Identification hit lists produced by CATS software (Camag) for an analyte fraction on two TLC systems in broad-scale screening analysis for drugs in liver. The reports were obtained by comparing the hR_F^c values (window ± 9 units) and *in situ* UV spectrum correlation against a library of 325 drug substances on each system.

visualization sequence, and the interpretation of the colour patterns is performed with a help of the Toxi-Lab Drug Compendium showing indexed colour photograms for hundreds of compounds. The sequence consists of formaldehyde vapour + Mandelin's reagent, water, fluorescence under 366 nm UV light and modified Dragendorff's reagent. There are also procedures and tests available for specific substances and classes of substances, such as opiates and cannabis. There is ample literature available on the applications of Toxi-Lab to clinical and forensic toxicology.

Another TLC screening scheme, Spot Chek (Analytical Bio-Chemistries, PA, USA), relies on a single mobile phase, a set of visualization reactions, and computerized interpretation of the patterns. Acidic/neutral and basic drugs are developed on separate plates, and on each plate the sample is divided into two or three equal portions that are developed in parallel to facilitate the use of visualization reactions. The migration distance is divided into five R_F zones with reference compounds. The computer program database is based on nine colour reaction responses and the plate zone locations for 243 drug substances but requires entry of only one TLC property to generate a matching list.

Automated Multiple Development

There are currently two alternative instrumental means to improve the Separation number (SN) in TLC: automated multiple development (AMD) and overpressured layer chromatography (OPLC). In AMD, the plate is developed repeatedly in the same direction, and each partial run goes over a longer solvent migration distance than the previous one. Each partial run uses a solvent of lower elution strength than the previous one and in this way a stepwise gradient is formed. SN values of up to 40–50 can be obtained by AMD but a disadvantage is the time required for the analysis, which may be several hours. There are no strictly forensic toxicological applications of AMD in the literature but the technique has an established position in the broad-scale screening for pesticides in the environment and has great potential in toxicology.

Overpressured Layer Chromatography

OPLC is based on the forced flow of the mobile phase against an external pressure, which results in short development times and decreased diffusion of the analyte fractions, making it possible to take advantage of longer developing distances. Silica gel plates are exclusively used in OPLC as reversed-phase chromatography has become complicated. Method development may be laborious due to disturbing adsorption zones, often obtained with multicomponent mobile phases. A commercial OPLC instrument is available from OPLC-NIT (Budapest, Hungary). OPLC has found use in the separation of closely related compounds in a particular pharmacological category or compounds originating from a particular botanical source. Two complementary OPLC systems have been developed for broad-scale screening for basic and neutral drugs with SN values close to 30, which is more than twice the values obtained typically with ordinary TLC: trichloroethylene–methylethylketone–*n*-butanol–acetic acid–water $17 + 8 + 25 + 6 + 4$, and butyl acetate–ethanol–tripropylamine–water $85 + 9.25 + 5 + 0.75$, with layer pre-saturation.

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Target Analysis

Drugs of Abuse

Amphetamines and related stimulants, especially amphetamine, methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA, an Ecstasy

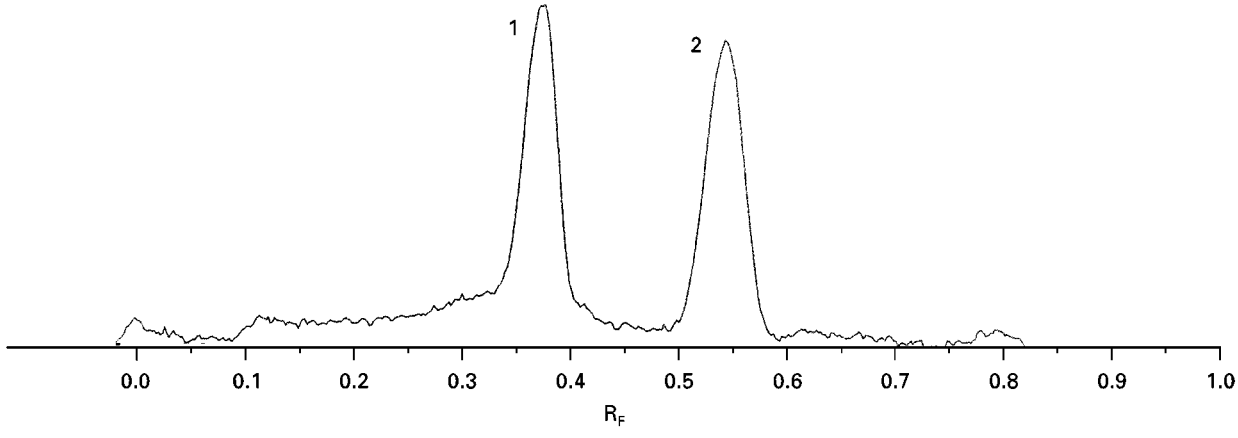

Figure 2 Separation of (1) methamphetamine and (2) amphetamine on the TLC system 6 of **Table 1**.

component), can be separated by a variety of TLC systems, such as those in Table 1 (**Figure 2**), and sensitively detected as fluorescent derivatives of, for example, fluorescamine or NBD-Cl (4-chloro-7-nitro-2,1,3-benzoxadiazole). Ninhydrin is a traditional reagent for amphetamines. Fast Black K salt reagent is capable of differentiating aliphatic primary and secondary amines, giving violet and orange-red colours, respectively, while tertiary amines do not react. Thus amphetamine and methamphetamine, or MDMA and 3,4-methylenedioxyamphetamine, are readily separated. Amphetamines possess poor UV characteristics, so they cannot be analysed by UV densitometric methods at low levels without derivatization. However, the methylenedioxy-derivatives can be easily recognized by their UV spectra (**Figure 3**). Despite the low limits of detection obtained with pure amphetamine-like substances, the limits of detection in urine are of the order of 250–500 ng mL^{-1} which is close to the standard immunoassay cutoff value (300 ng mL^{-1}).

The analysis of the main urinary cannabinoid, 11-nor-delta^9-tetrahydrocannabinol-9-carboxylic acid (THCA), is usually carried out with dedicated TLC systems, such as ethyl acetate–methanol–water–conc. ammonia 12 + 5 + 0.5 + 1. The detection of THCA is performed with various diazonium salts, such as Fast Blue B salt, Fast Blue BB salt and Fast Blue RR salt. Detection limits of 2–10 ng mL^{-1} can be obtained for THCA, and these concentrations compare favourably with the standard immunoassay cutoff level of 20 ng mL^{-1}.

Screening for cocaine is usually based on the detection of its metabolite benzoylecgonine (BE) in urine. The combination of the following two mobile phases can be applied to the separation: methanol–chloroform–ammonia 60 + 60 + 1, and ethyl acetate–methanol–water–ammonia 85 + 13.5 +1 + 0.5. The detection of BE is performed with Dragendorff's reagent or Ludy Tenger reagent, followed by sulfuric acid, with the limit of detection of 200 ng mL^{-1} in urine. The standard immunoassay cutoff level is 300 ng mL^{-1}

The opiates of interest in drug abuse testing programmes include the heroin metabolites, 6-monoacetylmorphine and morphine, and codeine. The combination of the following two mobile phases can be applied to the separation: ethyl acetate–isopropyl alcohol–methanol–ammonia 80 + 15 + 3 + 8, and 1,2-dichloroethane–isopropyl alcohol–methanol–ammonia 20 + 20 + 20 + 7. The limit of detection for opiates with iodoplatinate ranges from 100 to 500 ng mL^{-1} in urine, depending on the compound, while the standard immunoassay cutoff level is 300 ng mL^{-1}.

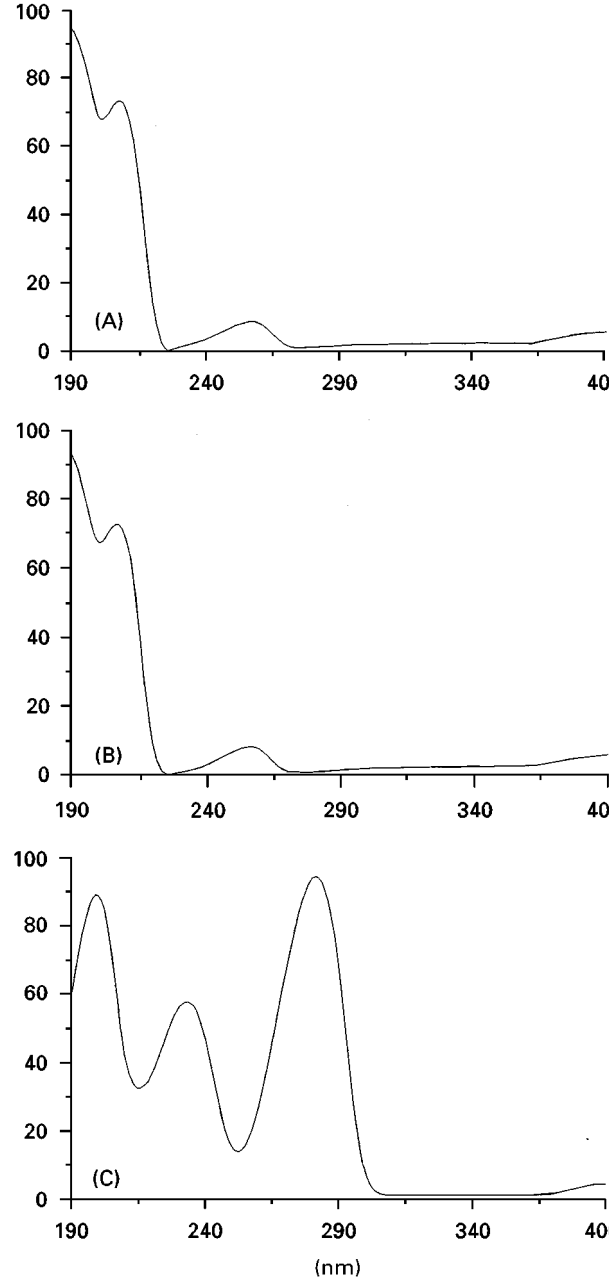

Figure 3 The *in situ* UV spectra of (A) amphetamine, (B) methamphetamine and (C) methylenedioxymethamphetamine (MDMA). Amphetamine and methamphetamine have similar spectra but they can be differentiated, e.g. by using the Fast Black K reagent.

Other Substances

Toxicological Analysis by Müller lists 1453 published TLC systems for potentially toxic compounds. A TLC bibliography is available from Camag on CD-ROM, listing 5500 abstracts of papers from 1982 to 1996. The biennial TLC reviews by Sherma in *Analytical Chemistry* provide a wealth of information on systems for individual substances in forensic toxicology.

Status of TLC in Forensic Toxicology Laboratory

In forensic toxicology, the unique features of TLC are best utilized in the broad-scale screening analysis for drugs and poisons in urine or liver samples. For this application, there are equipment, dedicated software and reference libraries available from several manufacturers. Compared to HPLC or capillary electrophoresis, TLC allows the detection of even poorly UV-absorbing compounds using selective visualization reactions. Compared to GC or GC-MS, TLC allows the chromatography of polar compounds without prior derivatization. Another important application of TLC is the screening or confirmation of drugs of abuse, although the supremacy of the combination of immunoassay and GC-MS in this area has hindered the development of modern dedicated TLC methods. Immunoassay screening, however, is vulnerable to sample adulteration and high background noise. In larger, broad-service laboratories, the various techniques available today, including TLC, are considered complementary rather than exclusive.

See also: **II/Chromatography: Thin-Layer (Planar): Modes of Development: Conventional; Modes of Development: Forced Flow, Over Pressured Layer Chromatography and Centrifugal; Spray Reagents. III/Alcohol and Biological Markers of Alcohol Abuse: Gas Chromatography. Clinical Chemistry: Thin-Layer (Planar) Chromatography. Clinical Diagnosis: Chromatography. Forensic Sciences: Capillary Electrophoresis.**

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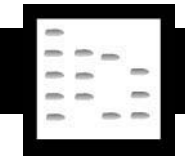

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Introduction

It is difficult to distinguish between aromas, flavours, taints and perfumes, because to a large extent these are artificial categories that overlap. An aroma may be defined as the smell emanating naturally (possibly in the process of cooking or other method of preparation) of a foodstuff or beverage. The aroma from coffee beans on roasting is a prime example but there are many others. The main criterion is that the aroma material is essentially all in the vapour phase and the nose is responsible for sensing the aroma. A flavour is intimately related to an aroma but may contain involatile compounds that give rise to the sensation of taste but in practice it is common to have a flavour with an associated aroma. A tainted foodstuff or beverage is often unsatisfactory for consumption because there are compounds present that have an unpleasant smell or taste. Taints may arise from natural